47

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THE GENERAL SOIL FLORA

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THE BACTERIAL FLORA OF FOUR SOILS COMPARED BY THE DIRECT MICROSCOPIC METHOD

LIDA M. THATCHER AND H. J. CONN

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TECHNICAL BULLETIN No. 129

THE GENERAL SOIL FLORA[1]

H. J. CONN

ABSTRACT

Methods by which the general soil flora has been investigated are discussed. It is pointed out that the methods in use in the past have not yielded significant results. The method used by the writer and those recently employed by Winogradsky in France are discussed; the important problems in soil bacteriology which remain unsolved are outlined; and the possible use of new methods in solving them mentioned.

INTRODUCTION

The study of the general micro-flora of soil is an intensely complex subject. In the first place, it is evident that practically all kinds of bacteria stand a chance of being introduced into the soil at some time. Whether or not any particular organism can be found there by an investigator hunting for it depends primarily upon whether that form has found conditions in the soil favorable for its development. In other words, the nature of the flora in any soil is determined by the conditions for growth present in that soil and not by the inoculation which it has received.

This fact, however, does not simplify the problem of studying the soil flora but rather tends to complicate it. The conditions in soil—physical, chemical, and biological—are capable of extreme variation, not only between different soils but in the same soil from time to time. That the differences in physical and chemical coditions may have their effects upon the types or organisms which predominate is almost self-evident, but that the nature of the flora depends upon biological conditions as well is less readily understood. This fact means that there is a complicated interrelation between the different types composing the soil flora. Many kinds of microorganisms capable of surviving the physical and chemical conditions present in any soil seldom get the chance to develop under natural

[1]Paper presented at symposium on "Soil Microbiology" at the joint meeting of the Society of American Bacteriologists and Section O of the American Association for the Advancement of Science, Philadelphia, December 29, 1926.

soil conditions, because they are ordinarily suppressed by other types still better adapted to the conditions in question. Some slight change in soil conditions, however, altho not necessarily enough to have much effect if the organisms were growing in pure culture, may completely upset the soil equilibrium, suppress the rivals of the organisms in question, and give the latter a chance to multiply. This interrelation between soil micro-organisms, and the resulting fluctuation in types with changing conditions, makes the study of the soil flora very complicated.

THE EARLY METHODS

Formerly, there were two distinct methods of investigating soil bacteria, one called quantitative and the other qualitative. It is a question whether either of these terms was correctly applied. The quantitative methods consisted wholly of making counts by the plate technic of the bacteria producing colonies on ordinary media. Recent work shows that these counts represent such a small fraction of the total soil flora that they hardly deserve the name quantitative. It has, in fact, long been realized, even by those using these methods, that such counts have little significance; and they have come to be used less and less as bacteriologists have learned more about bacteria in the soil

The so-called qualitative methods, on the other hand, consisted of the isolation of certain kinds of bacteria from soil and their study in pure culture. Such study was confined almost exclusively to the bacteria concerned in the nitrogen transformations of soil. These studies gave a very poor qualitative picture for various reasons, *viz.*, that there was never any positive knowledge that the bacteria thus investigated would behave in soil the same as in the laboratory culture media; there was no method of determining how much their behavior in pure culture would be modified by the presence of other bacteria in soil; and, furthermore, it was generally admitted that the particular organisms selected for this work were only a small part of the total soil flora, so it was not known how important they might actually be in any soil under investigation. In other words, it was quite well understood that little was known about the general soil flora. The same fact is true today, but methods are at last available by means of which its study seems possible.

The first methods that were proposed for obtaining more general information than by the procedures just discussed proved of com-

paratively little value, however, and have been almost given up today. The methods in question are those generally known as the Remy-Löhnis technic,[2] together with its various modifications. This technic is based on the assumption that if soil is added to a medium suitable for some particular nitrogen change, it is possible to measure the ability of the organisms in the soil to produce that change by determining the extent of the reaction taking place in the medium. The original media used in this technic were fluids, but it was soon realized that they offered conditions so different from the natural environment of soil bacteria that no significant results could be expected. Accordingly, various modifications of the technic were proposed which called for soil instead of culture-fluids as a substratum. It has been realized in recent years that even these modifications do not give results of real value, largely because the bacteria are being studied in mass and under conditions that are bound to be rather artificial. It is not at all impossible that procedures of this type may come into general use again later as auxiliary methods; but it has become quite evident that, used alone, they are not of as great value as their originators hoped.

At the time that the Remy-Löhnis method was being introduced, a piece of work of an entirely different nature was undertaken by Hiltner and Störmer.[3] This work did not go far, but it pointed the way toward the soil flora studies which, in the writer's opinion, seem at present to offer the greatest hope for the future. By members of the Remy-Löhnis school, Hiltner and Störmer's work was quite generally misunderstood. It was thought to be a mere quantitative study of soil bacteria, and hence a step backward instead of one in advance. To be sure, they did make bacterial counts, but the counts were determinations of numbers of different *kinds* of bacteria rather than of the total number growing on culture plates. They accomplished this in two ways: First, by a classification of the colonies on gelatin plates; and second, by a dilution technic for determining the numbers of nitrifiers, denitrifiers, nitrogen-fixing organisms, etc., present in their soils. This method of attack was

[2]Remy, Th. Bodenbakteriologische Studien. *Centbl. f. Bakt., 2 Abt.*, **8**, *657, 699, 728, 761. 1902.* Löhnis, F. Ein Beitrag Zur Methodik der bakteriologischen Bodenuntersuchung. *Centbl. f. Bakt., 2 Abt.*, **12**, *262–267, 448–463. 1904.*

[3]Hiltner, H. A., and Störmer, K. Studien über die Bakterienflora des Akerbodens, mit besonderer Berücksichtigung ihres Verhaltens nach einer Behandlung mit Schwefelkohlenstoff und nach Brache. *Kaiserl. Gesundheitsamt, Biol. Abt. Land. u. Forstw.*, **3**, *445–545. 1903.*

not followed up by anyone else and its possibilities never seem to have been fully appreciated.

The nearest approach to this same line of investigation was carried on by Chester[4] at about the same time and apparently independently. Chester's method consisted of isolating the predominating types from gelatin or agar plates and determining the extent to which these cultures were able to produce the end products of certain physiological activities. By this method, however, he did not necessarily classify or identify the species. By multiplying their numbers with the extent of their activity, he obtained a coefficient that was supposed to represent the "zymotic efficiency" of the soil. Chester's method had no practical application as he left it and, altho he pointed out certain valuable methods of attack, no one has developed the method further.

SOIL FLORA STUDIES

The soil flora studies of the writer began about 1909 and approached the subject from still a different angle. The original effort was to isolate from different soil samples enough cultures to get a good representation of the entire flora developing upon culture plates, then to study and classify these forms by the methods that had been worked out for classifying bacteria from other sources. Progress was discouragingly slow, and the first comprehensive publication on this subject (in 1917)[5] scarcely went further than to point out the predominating groups of bacteria in the soil, so far as these could be obtained from culture plates. The most discouraging feature about this work was the apparent similarity between the flora of different soils and between that of the same soil under varied conditions. It was realized finally that the chief obstacle preventing progress was a lack of methods, as the means by which bacteria of other sources could be classified did not give satisfactory results in the case of those from soil. Accordingly, for several years, more attention was given to technic than to the actual study of the soil flora. It is apparent now that this digression into the field of technic was worth while.

The first real contribution of importance coming from this study

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[4]Chester, F. D. Studies in soil bacteriology. *Delaware Agr. Exp. Sta. Rpt.*, **14**, *46–78. 1903.*

[5]Conn, H. J. Soil flora studies, I–V. *New York Agr. Exp. Sta. Tech. Buls. Nos. 57 to 60. 1917.*

of methods was the development in 1917 of a direct method for studying bacteria in soil by means of microscopic examination.[6] This method was realized from the start to be a valuable piece of technic, but its use in connection with soil flora studies was not appreciated at the time. When the technic was published, it was hoped that it would prove suggestive to others and that someone else might find a method of using it. This is exactly what proved to be the case, and an application of it has been proposed by that veteran soil bacteriologist, Winogradsky,[7] formerly of Russia, now located in France.

The second important step in the way of technic was the developing of methods for studying and classifying the non-spore-forming bacteria of soil. This group of organisms has proved puzzling from the start, so much so that in general they have been neglected entirely by soil bacteriologists except for those few members of the group that take part in nitrogen changes. A large majority of the non-spore-forming bacteria in soil are very small, slowly liquefying rods which grow so poorly in ordinary bacteriological media that they cannot be told one from another by means of the usual cultural and physiological reactions. As a result, their study and classification has proved extremely baffling. It was finally learned, however, that if they are grown in certain synthetic media, especially solid media, it is possible to obtain physiological distinctions between them which do not show up on the usual substrata. It was further learned that these organisms are not so nearly alike morphologically as was formerly supposed. In the earlier work, their morphology had been studied uniformly in 24-hour culture. As it began to be realized that bacteria may show morphological changes from day to day, the procedure was adopted of following up the morphology of each culture for a week before regarding it as completely described. By this means it proved possible to recognize certain morphological differences between the cultures which correlate sufficiently with the physiological characteristics of the organisms to appear to represent natural groups suitable for classification.[8] The possibility of

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[6]Conn, H. J. The microscopic study of bacteria and fungi in soil. *New York Agr. Exp. Sta. Tech. Bul. No. 64. 1918.*

[7]Winogradsky, S. Sur l'étude microscopique du sol. *Compt. Rend. Acad. d. Sci., Paris,* **179**, *367–371. 1924.*

[8]Conn, H. J. Soil Flora Studies, VI. The punctiform-colony-forming bacteria in soil. *New York State Agr. Exp. Sta. Tech. Bul. No. 115. 1925.*

classifying these little-understood organisms is now pointing out methods of attack for their further study.

THE DIRECT MICROSCOPIC METHOD

Quite recently a much more valuable contribution has come from the laboratory of Winogradsky.[9] This investigator, as just mentioned, has done what it was hoped some soil bacteriologist would do, namely, tried out the direct microscopic method of studying soil bacteria and applied it to a new procedure for studying the general soil flora. The technic itself he has modified slightly, but not in any essential particular. His invaluable contribution is to show a method of using it. He points out that any factor which changes the normal soil conditions, such as the addition of some nutrient material, is likely to cause the stimulation of some one or two kinds of microorganisms. It is possible to observe their increase in numbers by means of microscopic preparations and in this way to obtain valuable information concerning the biological reaction of any soil to the introduction of any added material. By means of auxiliary culture plates made at the proper times, it is very often possible to pick out the organism thus stimulated and in this way to obtain a more accurate picture as to the behavior of the general soil flora than has been obtained by previous methods.

Using this technic, Winogradsky has shown that there are two large categories of bacteria in soil. The first of these he calls the *autochtonous* flora, and finds it to be generally present in soils that are poorly supplied with fermentable substances. These bacteria are comparatively inactive, presumably taking part in the slow combustion of the humic constituents of the soil. The other category he calls the *zymogenic* organisms. The latter are not so abundant in normal soil as the autochtonous forms and are stimulated to activity only by the presence of readily fermentable material. Therefore, their growth is intermittent, periods of intense activity being followed by periods of rest. Several years ago the writer, in collaboration with J. S. Joffe,[10] in discussing one group of these zymogenic forms, namely the spore-formers, stated that their usual rôle in soil

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[9]Winogradsky, S. Études sur la microbiologie du sol: I. Sur la méthode. *Ann. Inst. Pasteur*, **39**, *299–354. 1925.* II. Sur les microbes fixateurs d'azote. *Id.*, **40**, *455–520. 1926.*

[10]Joffe, J. S., and Conn, H. J. Factors influencing the activity of spore-forming bacteria in soil. *New York State Agr. Exp. Sta. Tech. Bul. No. 97. 1923.*

is one of "watchful waiting." Winogradsky quotes the expression without translating it and declares that it gives a very suggestive picture of the actual state of affairs.

Winogradsky shows further that the microscope may be used to pick out the types of zymogenic organisms which develop in a soil under various conditions. Thus, he supplies a method for learning more about the actual behavior of bacteria in soil than can be obtained by any of the indirect methods previously in use. His procedure is without question the most valuable contribution yet made to the methods of studying the general micro-flora of soil.

Winogradsky's methods, with slight modification, are being put into use in the writer's laboratory in the following manner. A series of four different soils have been selected for comparison, one a highly productive soil and the other three soils of rather low productivity. A comparison is obtained between these four soils by removing 50-gram samples from each and mixing with some ingredient which can serve as a food for bacteria. So far the ingredients selected have been mannite, peptone, and starch. These samples are thoroly moistened, but not enough to puddle or to saturate the soil, and are incubated in petri dishes under a bell jar at room temperature. Every day or two after adding these materials to the soils, small samples are withdrawn and studied by the direct microscopic method. The difference in the flora developing in the case of the different treatments is very striking. Following the addition of mannite, as already pointed out by Winogradsky, there is a tendency for the development of types which seem to be *Azotobacter*. When peptone is added there is always an increase of spore-forming rods, with the especially surprising fact that all of the soils studied have invariably shown the presence on the eleventh day (but almost never earlier) of a striking polar-spored rod. The reaction to starch is noticeable but not so striking. Such facts have already been pointed out by Winogradsky, but the present investigation is not so much intended to verify his findings as to learn how the four soils in question compare by these methods.

This work has not yet progressed far enough to make definite statements. It does show, however, that differences can be established in this way between different soils that have not been brought out by any method of comparison previously adopted. The method, therefore, seems to be workable and to be full of promise.

UNSOLVED PROBLEMS

After this brief survey of the earlier work on the soil flora and of the methods by which the subject is being attacked at present, the question arises, What are the principal problems in this line calling for a solution in the future? There are, of course, many such problems; and the further the work progresses, the more we shall be faced with unsolved problems to attack. In short, the field is largely unexplored. It is obvious that all of these problems cannot be attacked at once and that the exact nature of many of them at present cannot be anticipated. The best we can do in outlining the future problems in this work is to point out those which seem now to be most pressing and to promise most interesting results.

In the writer's estimation there are two problems that stand out in this way. The first of these relates to the function of the little-understood non-spore-forming organisms in soil which compose a large part of the autochtonous flora of Winogradsky. Now that it is possible to make a preliminary classification of this group, we can select different types for more intensive investigation. The abundance of such organisms as these in the soil suggests that a knowledge of their function should be of importance in the study of soil bacteriology. The second most pressing problem is to see how different soils compare by the direct method of Winogradsky, using the technic just outlined. By using these two methods of attack and by harmonizing the results, it is hoped that the next few years will show actual progress in studying the general soil flora.

THE BACTERIAL FLORA OF FOUR SOILS COMPARED BY THE DIRECT MICROSCOPIC METHOD

LIDA M. THATCHER[1] AND H. J. CONN

ABSTRACT

Four soils, known in the Bureau of Soils Classification as Dunkirk silty clay loam, Dunkirk fine sand, Volusia silt loam, and Hoosick coarse sandy loam, respectively, were studied by the direct microscopic method, using some of the modifications of this technic recently suggested by Winogradsky.

The response of the bacteria in these soils to the addition of peptone, starch, mannite, and soybean meal (with and without liming) was studied by means of the microscope.

The principal differences thus observed correlated with the reaction of the soils, the two acid soils (those of the Volusia and Hoosick series) responding to treatment in a distinctly different manner from the other two soils. This difference was partly, but not wholly, overcome by the addition of calcium carbonate.

Altho no striking conclusions concerning the soils studied are brought out by this investigation, it is suggested that the method employed may have value and is worth using in the study of other soils.

INTRODUCTION

The preceding paper, written six months before the completion of the work outlined below, serves as an introduction to the present investigation, and explains the reason for undertaking it. It is enough here to add that Winogradsky's modification of the microscopic technic originally proposed by one of the authors seemed to promise a method of observing differences between the bacterial flora of soils not brought out by other methods. One of the chief weaknesses of soil bacteriological investigations has always been the failure of the methods employed to demonstrate differences between the kinds of bacteria in soils known to be quite unlike in physical, chemical, and crop-producing characteristics. Winogradsky's observations, however, indicate that this apparent similarity may be due to the fact that all soils contain essentially the same

[1]Technical Assistant, Commission on Standardization of Biological Stains, Geneva, N. Y.

"autochtonous" flora, altho they may differ distinctly in the types of organisms normally present in small numbers but capable of rapid multiplication under suitable conditions. His methods, therefore, are designed to show differences in the potential as well as actual flora of any soil under investigation. For this reason it was hoped that they might bring to light differences in the nature of the flora of certain local soils, altho these same soils had previously been investigated by other methods without such differences being evident. The present work was planned, therefore, with the primary object of studying the practical value of the technic, but with the hope that it might also furnish interesting information about the soils to which the technic was applied.

THE TECHNIC

As originally developed by one of the authors,[2] the technic for the microscopic examination of soil is as follows: Make a suspension of soil in nine times its weight of a 0.015 per cent solution of gelatin. (The exact strength of the gelatin solution is quite important. If too strong it coats the soil granules with a film which stains the same color as the bacteria, while if too weak it will not adhere properly to the glass.) Smear a drop of this suspension in a thin film on a slide. (Both the amount used and the size of the smear must be measured if the number of bacteria is to be counted.) The film is then dried on a flat surface over a boiling water bath. Without removing from the water bath, the slide is flooded with a 1 per cent phenolic solution of rose bengal, and the stain is allowed to act for one minute. At the end of this period the slide is washed very briefly by immersion in water and replaced on the water bath to dry.

These preparations should be examined with an oil-immersion objective and a high-power eye-piece. The combination of lenses found to be most satisfactory is a 1.9 mm (1/12 inch) fluorite objective (numerical aperture of 1.32) with a 12.5X ocular. When counting bacteria, it is advisable not to count the entire field, but to have the central portion of it marked off as described by Breed and Brew[3] for milk work. They used a disc in the eye-piece with circles and cross-lines ruled on it so as to limit the area within which the bacteria were

[2]Conn, H. J. The microscopic study of bacteria and fungi in soil. *New York Agr. Exp. Sta. Tech. Bul. No. 64. 1918.*

[3]Breed, R. S., and Brew, J. D. Counting bacteria by means of the microscope. *New York Agr. Exp. Sta. Tech. Bul. No. 49. 1916.*

counted. A similar arrangement may be used for soil. It has been found convenient to use a circle of such a size as to cover an area on the slide either 80 microns or 113 microns in diameter. Each organism in the former area represents approximately 2,000,000 bacteria per cc of the soil infusion, while each organism in the latter represents 1,000,000 per cc. If the soil infusion has been diluted 10 times, these factors should be multiplied by 10, i. e., 20,000,000 and 10,000,000 per cc of soil infusion.

It is important that a good source of illumination be used in this work. The brightest daylight can be used, but a better and more constant source of light can be obtained by artificial means, electric light filtered thru "daylight" glass giving very satisfactory results. Minute organisms may easily be overlooked if the light is not good.

WINOGRADSKY'S MODIFICATION

In his use of the direct microscopic method of studying soil flora, Winogradsky[4] has modified slightly the technic given above. His methods are as follows: One gram of finely divided soil, taken from a well-mixed sample, is shaken up in 4 cc of distilled water for 5 minutes. After settling for 30 seconds, the liquid is decanted into a small hand centrifuge tube. Two successive lots of 3 cc of water are added, shaken for 1 minute, and allowed to settle for 30 seconds, finally being decanted into the same tube. Thus, 10 parts of water in all are used for 1 part of soil. During this manipulation, which takes about 10 minutes, a deposit is formed in the centrifuge tube. Without disturbing the latter, half of the supernatant liquid is poured into a second tube and centrifuged until the liquid ceases to become clearer. There are then three deposits and two different suspensions, one having been centrifuged and the other not. Five preparations are made from them, using approximately equal quantities of deposits and liquids, and smearing over equal surfaces of the slides. After drying, the soil particles are fixed to the slide, using a warm 1 per cent agar solution for the first and second deposits and a cold 0.1 per cent agar for the third. In the case of suspensions which contain a basis of colloidal matter encrusted with minute particles, it is unnecessary to use any fixative. When dried, the slides are treated with absolute alcohol for 1 to 2 minutes, after which they are again dried and stained in a phenolic solution of erythrosin

[4]Winogradsky, S. Sur l'étude microscopique du sol. *Compt. Rend. Acad. d. Sci., Paris*, **179**, *367–371. 1924.*

which Winogradsky prefers to rose bengal. The stain is allowed to act cold from 5 to 15 minutes, after which the slides are washed and examined in water.

In the present investigation the authors were not so much concerned with the microscopic technic of Winogradsky as they were with his application of the direct method to the studies of the general soil flora as modified by soil treatment. It seems to the writers that Winogradsky's most valuable contribution lies in those experiments in which samples of soils were treated with different materials in the laboratory, the subsequent changes in the bacterial content of the soil being traced by microscopic examination.

PRESENT MODIFICATION AND APPLICATION

Recently a method for staining the bacteria in soil was developed by one of the authors,[5] differing from the original technic in two respects. It was this later method which was used in the work herein reported. The technic is as follows, the italics showing the points in which it differs from the original procedure: Make a suspension of soil in nine times its weight of a 0.015 per cent solution of gelatin. Smear a drop of this in a thin film on a slide. (Both the amount used and the size of the smear must be measured if the number of bacteria is to be counted.) *Immerse for from 1 to 3 minutes in a 40 per cent solution of acetic acid or a N/10 solution of hydrochloric acid. Wash off the excess acid briefly,* and dry on a flat surface over a boiling water bath. While still on the water bath, cover the film with a 1 per cent *aqueous* solution of rose bengal, phloxine B, or erythrosin Y, and allow it to stain for about 1 minute. The authors prefer rose bengal to the other dyes.

The application of the direct method in the present investigation was similar to that of Winogradsky, namely, in the study of the bacteria present in soils which had previously been treated in various ways. Microscopic preparations were made on successive days from the same samples to show what change, if any, had taken place in the population of the soil, either in numbers or character of the organisms present.

Two methods of handling the soils in the laboratory were adopted. The major portion of each sample was stored in a large wooden box, the soil being well sprinkled with water from time to time to prevent

[5]Conn, H. J. An improved stain for bacteria in soil. *Stain Technology*, **1**, *125–128. 1926.*

drying and caking, while smaller portions, 300 to 500 grams, were sifted thru a fine sieve, moistened sufficiently, and kept in large glass bottles. It was found that considerable time could be saved in setting up the different experiments if this previously sifted, moistened soil was used, altho frequently samples were taken from the larger boxes.

In setting up the various experiments, small quantities, usually 100 grams, of each soil were sifted and mixed thoroly with the test material. After being placed in a petri dish, each sample was moistened with a known quantity of water, care being taken to prevent puddling of the soil. The plates were then incubated under a bell jar, which contained, also, a beaker of water to keep the air moist. When a decrease in the total number of bacteria in the soils indicated a lack of proper moisture content, a known quantity of water was added to each plate and the microscopic study continued.

Record was kept of the relative numbers of large and small coccoid forms, long and short small rods, bacterial spores, and fungus growth present in the preparations.

THE SOILS USED

To learn whether the technic thus applied might have any relation to the productivity (or any other characteristics) of different soils, four distinctly different soil types were selected for study. One of them (Dunkirk silty clay loam) was a soil of relatively high productivity, while the others were moderately unproductive or at least required special handling in order to secure good crops. For convenience, these samples were denoted by letters standing for the soil types as classified by the Bureau of Soils. The samples were as follows:

Sample DC. Soil from the Experiment Station farm at Geneva of the type classified as Dunkirk silty clay loam. The sample was taken from the edge of a cultivated field which had borne various crops successively for a number of years. The soil is distinctly alkaline in reaction and is of such high natural productivity that it ordinarily shows little response to the addition of fertilizer.

Sample DS. Soil from the edge of an orchard about 5 miles out of Geneva, in the region known as Pine Plains, of the type classified as Dunkirk fine sand. It is an extremely light sand of reddish yellow color. The soil is about neutral in reaction and produces rather poor crops. Soil of this type, transferred to the greenhouse, does not seem to show much response to fertilization and does not produce

good yields, even tho the necessary nutrients are added and the reaction is corrected.

Sample V. Soil from a hill on the east side of Canandaigua Lake, about 3 miles due west of Rushville, from a spot denoted in the Soil Survey of Ontario County[6] as being Volusia silt loam. The sample was taken from an uncultivated and almost bare strip beside a road. This soil, like the others of the Volusia group, is very acid in reaction and is of low productivity even when the reaction is corrected. These soils do not ordinarily seem deficient in plant nutrients, but they are characterized by the very slow rate at which organic matter decomposes, whether lying on the surface or mixed into the soil.

Sample H. A sample of field soil from Kinderhook, N. Y., in the Hudson River Valley, which had been undisturbed for years and bore its natural flora. This soil belongs to the type classified by the Bureau of Soils as Hoosick coarse sandy loam and is distinctly acid in reaction. Soils of this type bear a very scanty growth in their natural state, consisting mostly of the plants suited to acid conditions; but after cultivation they are capable of good yields, particularly if limed. It is difficult to maintain their humus content, but for a different reason than in the case of the Volusia soils. In the Hoosick soils organic matter decomposes rather *too* rapidly, and very frequent turning under of cover crops is necessary to maintain productivity.

Before undertaking the present investigation some interesting observations on the bacterial flora of this last type of soil had been obtained which bear on the above-mentioned characteristics. Several samples of this soil from a cultivated orchard had been studied and had been found quite normal in their bacterial flora, as judged by microscopic study and by means of gelatin plates. The present sample, however, was found, when first received, to have an unusually low bacterial content, about 1,000,000 per gram or lower, as judged by the plate count, and too low for satisfactory microscopic study. Upon standing without treatment in the laboratory, however, a bacterial flora gradually developed which compared favorably in numbers with that of the other soils studied in this work. This increase in numbers occurred too slowly to be the direct result of increased aeration on the growth of the micro-organisms, but suggested, rather, that the aeration slowly made available for the bacteria certain food material in the soil which had been previously unavailable. When the work reported below was carried out, the

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[6]Soil Survey of Ontario County, New York. *United States Dept. Agr., Bureau of Soils. 1912.*

soil had been in the laboratory about a year, and the new flora had apparently become well established.

EXPERIMENTAL

It was felt that if different soils studied by the technic described above could give results correlating with productivity, or with their physical and chemical characteristics, these four soils should give indication of such a correlation. Sample DC was highly productive without special treatment, while the others were non-productive unless specially handled, altho soil H is capable of yielding splendid crops. Samples DC and DS were alkaline or neutral in reaction; samples H and V distinctly acid. Sample DC was a clay loam and sample DS a fine sand, while samples H and V were intermediate in texture.

With such striking differences between the soils it was believed that their reactions to treatment should be distinct. Accordingly, four experiments were carried out by the direct microscopic technic, and subsequently, a few supplementary tests were made to throw light on certain questions raised by the first experiments.

DIRECT MICROSCOPIC METHOD

EXPERIMENT I

Following the line of Winogradsky's investigations on the effect upon the bacteria in soil of the addition of various substances, 16 plates were set up, 4 from each of the four soils under observation. Three samples in each set were treated with 2 per cent peptone, 2 per cent mannite, and 2 per cent starch, respectively, while the fourth plate was left untreated to be run as a control. Microscopic preparations were made on the 2nd, 3d, 4th, 7th, 8th, 9th, 10th, and 12th days, 2 cc of water having been added to each plate on the 7th day.

A study of the tabulations made of the relative numbers of organisms present in the eight sets of slides prepared in this experiment showed the following results.

The *peptone-treated* samples of all four soils were characterized by the presence of numerous rods, both long and short small rods being present in considerable numbers; while sample DS showed, also, great numbers of large spore-forming rods which did not appear in the other soils to any appreciable extent. In all four soils a tetanus-like spore-former developed on the 10th and 12th days. The soils

with *mannite* showed a general increase in the numbers of large and small coccoid forms, especially in the case of samples H and V. The soils treated with *starch* showed the least growth of any, there being fewer organisms present in samples DS and DC than in the check samples of these soils. There was an increase in the large and small coccoid forms in the H and V preparations, greater numbers developing in the former sample. In all four soils there was a marked increase in the bacterial count after the addition of water to the samples which had become so dry that growth was inhibited.

No absolutely clear-cut differences were observed between the different soils in the way in which they responded to the various materials added. It was noticeable, nevertheless, that samples DS and DC showed great similarity in the types of organisms developing after any given treatment, while samples H and V (the two acid soils) were also very similar to each other but showed certain differences from the two soils that were not acid in reaction. The chief exceptions to this general rule were that with peptone sample DS alone produced great numbers of large spore-forming rods and sample H larger numbers of rods than sample V, while with mannite and with starch sample V produced more of the coccoid forms than sample H.

EXPERIMENT II

Since it was desirable to duplicate, if possible, results obtained in the previous experiment, another set of plates was made up from the soils under observation, again treating 100-gram samples with 2 per cent peptone, 2 per cent mannite, and 2 per cent starch, respectively. These were incubated as before and microscopic preparations made upon corresponding days.

Examination of the counts made from eight sets of slides showed a distinct correlation between the readings of these preparations and those made in the previous experiment. Again there seemed to be a decided similarity between the behavior of samples DS and DC, on the one hand, and samples H and V, on the other. In the case of the *peptone* treatment, samples DS and DC produced more rods than did the other two, with sample H giving rise to larger numbers than sample V. As before, all four soils showed the presence of tetanus-like rods which appeared in each case in the 11th day preparations. These rods showed up on the 10th and 12th days in the previous experiment. Treatment with *mannite* and *starch* again caused the development of greater numbers of large and small

TABLE 1.—TYPES OF BACTERIA DEVELOPING IN UNLIMED SOIL, SUMMARY OF EXPERIMENTS I AND II.

SOIL	CONTROL	MATERIAL ADDED		
		Peptone	Mannite	Starch
DS Dunkirk fine sand	Small numbers of all types of organisms present, especially small coccoid forms	Numerous long and short small rods; large numbers of large spore-formers; tetanus-like rods appeared on 12th day	Noticeable increase in large and small coccoid forms	Fewer organisms than in control plates
DC Dunkirk silty clay loam	All types in small numbers, especially short rods and small coccoid forms	Numerous long and short rods; tetanus-like rods appeared on 10th and 12th days	Increase in large and small coccoid forms	Fewer organisms than in control plates
H Hoosick coarse sandy loam	All types in small numbers, especially long small rods	Short small rods more numerous than long small rods; tetanus-like rods appeared on 10th and 12th days	Greater increase in large and small coccoid forms	Increase in large and small coccoid forms and short small rods
V Volusia silt loam	Equal numbers of all types, more organisms present than in other three soils	Long and short rods numerous; tetanus-like rods appeared on 10th and 12th days	Most decided increase in large and small coccoid forms	Most increase in large and small coccoid forms

coccoid forms in samples H and V than in samples DS and DC, with the larger numbers in sample V.

The results of Experiments I and II have been summarized in Table 1.

EXPERIMENT III

It was very noticeable that so far as any bacteriological differences between the soils were observed in this work they correlated with the reaction of the soil. This raised the question whether the difference might be due to actual dissimilarity in the bacterial flora of the soils rather than to the inability of certain bacteria present in every soil to develop in the presence of acid. To investigate this point a second set of plates was made up and run simultaneously with those in Experiment II. These plates were also treated with peptone, mannite, and starch but had in addition 1 per cent of $CaCO_3$ mixed thoroly with the soil. A set of control plates on the $CaCO_3$ alone was run at the same time.

Examination of the microscopic preparations made on successive days showed some further differences in the behavior of the four soils. Sample H, with *peptone*, from the 3d to the 8th day showed the presence of large numbers of very tiny rods which did not appear in any of the other soils, and sample DC developed several tetanus-like rods on the 7th day. In the samples treated with *mannite* there was not much change, except in the case of sample DS which showed numerous yeast cells and spores on the 8th, 9th, and 11th days. Following the *starch* treatment there were small coccoid forms present in small numbers in all of the preparations, while the only unusual feature was the lack of small short rods in sample H. These rods developed in each of the other three samples.

Here, again, as in the previous experiments, there seemed to be a decided tendency for sample DS to respond to treatment similarly to sample DC, while sample H resembled sample V. Evidently the difference between these two groups of soils is not wholly overcome by correcting the reaction of the two acid soils.

The results of this experiment are summarized in Table 2.

EXPERIMENT IV

In order to study still further the types of bacteria developing in soils under various treatments, a set of four plates was made up in which the soils had been treated with 2 per cent soybean meal.

TABLE 2.—TYPES OF BACTERIA DEVELOPING IN LIMED SOIL, EXPERIMENT III.*

SOIL	CONTROL, $CaCO_3$ ALONE	MATERIAL ADDED		
		Peptone	Mannite	Starch
DS Dunkirk fine sand	Numerous short rods; all other types present in equally small numbers	Numerous long and short small rods; large numbers of large spore-formers; bacterial spores numerous; tetanus-like rods on 11th day	Fewer organisms of all types; numerous yeast cells	Fewer organisms than in control plates; no small coccoid forms
DC Dunkirk silty clay loam	All types present in small numbers; short small rods most numerous	Large numbers of long and short small rods present; a few large spore-formers and spores; tetanus-like rods on 7th day	Numerous large yeast cells present; short small rods in small numbers; almost no long small rods	Fewer organisms than in control plates; all types present in equal numbers
H Hoosick coarse sandy loam	All types present in small numbers	Large numbers of very tiny rods 3d to 8th day; very few large spore-formers; no large coccoid forms present; several small coccoid forms	Very few organisms, chiefly short rods and small coccoid forms	No small short rods; few organisms present, chiefly large coccoid forms
V Volusia silt loam	A few short rods; no long small rods; numerous large and small coccoid forms	Numerous long and short small rods; no large rods; small numbers of large and small coccoid forms	More short rods and small coccoid forms than in other three soils; large coccoid forms	More organisms than in other three soils, chiefly large coccoids and short small rods

*This Experiment was set up and run simultaneously with Experiment II.

The usual procedure was followed, microscopic preparations being made on successive days and the results tabulated as before.

A study of the counts made indicated that this treatment gave results differing distinctly from those in previous experiments. All four soils were characterized by the presence of many small coccoid forms which were more numerous in samples DS and DC than in the acid soils. Samples H and V showed greater numbers of the large coccoid forms, but it was questionable whether these might not have been fungus conidia in the preparations made from sample H on the 7th to the 11th days. Sample H did not show more than a very few long small rods at any time, while the other three soils produced them in large numbers. Small short rods developed in all four soils, but again samples DS and DC gave rise to larger numbers than did samples H and V. Each of the four plates showed the presence of molds, the soil being completely permeated and bound together by the mold filaments.

A summary of the results of this experiment appears in Table 3.

One characteristic of the soils in this soybean meal series, evident to the naked eye, was the presence of molds in great abundance. The ordinary microscopic technic is not adapted for demonstrating fungi in soil, and, except for the numerous fungus conidia to be seen, the preparations made from these soils gave little evidence of the actual abundance of these organisms. They were so plainly thriving in these soils, however, that it was suspected that acid conditions might have developed.

EXPERIMENT V

A set of four plates was prepared similar to those in Experiment IV, containing 2 per cent soybean meal but with the further addition of 1 per cent $CaCO_3$. Microscopic preparations were made from the plates on various days as usual, and the results are summarized in Table 4.

The most striking feature in the results of this experiment was the lack of the small coccoid forms which characterized all four soils when treated with soybean meal alone. These coccoids had been more abundant in the DS and DC samples than in the other two, as related above. In the present experiment, however, there were fewer of these coccoid forms in all four samples, and while they were more numerous in sample DS, sample DC did not produce them in any larger numbers than did samples H and V. Sample DS was

TABLE 3.—TYPES OF BACTERIA DEVELOPING IN UNLIMED SOIL TREATED WITH SOYBEAN MEAL, EXPERIMENT IV.

DS, DUNKIRK FINE SAND	DC, DUNKIRK SILTY CLAY LOAM	H, HOOSICK COARSE SANDY LOAM	V, VOLUSIA SILT LOAM
Small coccoids more numerous than in H and V soils; numerous long small rods; greater numbers of short small rods than in H and V soils; soil permeated by molds	Small coccoids more numerous than in H and V soils; numerous long small rods; greater numbers of short small rods than in H and V soils; soil permeated by molds	Numerous small coccoid forms; large numbers of large coccoid forms; very few long small rods; small numbers of short small rods; soil almost completely bound together by mold filaments	Numerous small coccoid forms; large numbers of large coccoid forms (fungus conidia?);* numerous long small rods; small numbers of short small rods; soil permeated by molds

*It was not certain that these coccoid forms were fungus conidia, but there was considerable evidence that such was the case. If so, it is quite possible that the large numbers of large coccoids in sample H might also be conidia instead of coccoid forms of bacteria.

TABLE 4.—TYPES OF BACTERIA DEVELOPING IN LIMED SOIL TREATED WITH SOYBEAN MEAL, EXPERIMENT V.

DS, DUNKIRK FINE SAND	DC, DUNKIRK SILTY CLAY LOAM	H, HOOSICK COARSE SANDY LOAM	V, VOLUSIA SILT LOAM
Small coccoids more numerous than in other three soils; many more long small rods than in other three soils; tetanus-like rods present on 11th day; small short rods more numerous than in other three soils; large numbers of bacterial spores, three times as numerous as in any of other three soils; fungus growth present*	Small coccoids more numerous than in H and V soils, fewer than in DS soil; long small rods in equal numbers with H and V soils, less than in DS; fewer short small rods than in other three soils, more similar to H and V; very few bacterial spores; fungus growth	Numerous small coccoid forms; fewer long small rods than in DS and DC soils, equal numbers with V soil; short small rods more numerous than in DC, less than in DS, equal numbers with V soil; large numbers of fungus spores; no bacterial spores	Small coccoids in smaller numbers than in DS and DC, equal numbers with H soil; fewer long small rods than in DS and DC soils, equal numbers with H; more short small rods than in DC and H soils, fewer than in DS; numerous fungus spores; no bacterial spores

*As soon as one day after treatment all four soils were permeated by molds which disappeared almost entirely after four days. Fungus spores were present in preparations from samples H and V, but did not show up in the other two soils.

characterized by the presence of more organisms of all types and by large numbers of bacterial spores which were negligible in the other three samples. There was a decided similarity between the readings from samples DC, H, and V, very little, if any, difference being noticeable in their flora with the exception of the presence of large numbers of fungus spores in sample H. There were a few of these spores in sample V, but they were nowhere near as numerous as in the preparations from sample H.

SUPPLEMENTARY TESTS

The results of the above investigations having tended to place samples DS and DC in one category with samples H and V in another, certain further tests were performed to see if these observations correlated with any other characteristics of the soils as bacterial habitats.

One of the authors has recently become interested in a certain group of bacteria designated in a previous publication[7] as coccus-forming rods, because of their characteristic of living as rods for one or two days on a fresh substratum and then becoming spherical and resembling micrococci. It has been observed that this group of bacteria occurs most abundantly in the more highly productive soils so far investigated and is distinctly more numerous in cultivated soil than under sod. The characteristics of these organisms are to be discussed in a subsequent publication, but certain observations as recorded above led to an investigation of the four present soil samples as habitats for these bacteria.

The method employed in searching for bacteria of this type was to plate out the soils in plain gelatin (without added nutrients), to incubate for 7 days at 18°C, to isolate from each plate about 15 cultures of the punctiform colony type, and then to make microscopic preparations from the slants on the 1st, 3d, 5th, and 7th days. In this way it was possible to get an idea as to the relative abundance in the soils of those forms which are rods in young culture but become spherical after about three days.

Repeated tests of the Dunkirk silty clay loam made in this way showed that approximately two-thirds of the cultures isolated were coccus-forming rods; and as the punctiform colonies are ordinarily about 60 per cent of those developing on the plates made from this

[7]Conn, H. J. Soil flora studies, VI. The punctiform-colony-forming bacteria in soil. *New York State Agr. Exp. Sta. Tech. Bul. No. 115. 1925.*

soil, it may be roughly estimated that the coccus-forming rods constitute something like 40 per cent of the organisms that grow on the plates. In the case of the Dunkirk fine sand the proportion has ordinarily been found to be rather lower and since the total flora of this soil is quite scanty, the actual numbers of these organisms present in it must be much below those found in the clay loam.

The soils represented by samples H and V have not been studied as frequently as the Dunkirk silty clay loam, but samples of the Hoosick soil have been examined often enough to show that these organisms are almost, if not entirely, lacking. A test of sample V has shown the same lack of these organisms in that soil.

These observations again placed samples DS and DC in one category, and samples H and V in another, and they also suggested that the small coccoid bodies which developed in the two former soils when treated with soybean meal (Experiment IV) might be these coccus-forming rods occurring in their coccus state. As it was not known, however, whether these organisms appeared as rods or as cocci in any of the soils in question, the following two experiments were planned to investigate the point.

EXPERIMENT VI

Small quantities (about 10 grams) of each of the four soils were sterilized in test tubes at 15 pounds pressure for 20 to 30 minutes and inoculated with suspensions of 12-hour cultures of some organism of the group in question. The work was first carried out using only one culture, and was subsequently repeated with the same culture and with three others in addition, each of which was originally isolated from a different source.

Microscopic preparations were made from the 20 tubes thus inoculated on the 1st, 2nd, 4th, and 5th days after the inoculation. Two facts were strikingly brought out by the examination of the preparations, *viz.*, that the organisms appeared as small cocci even as early as the first day after inoculation, without the preliminary development of the rod forms observed on agar slants, and that, altho these small coccus-like forms were present in all 20 tubes, they were conspicuously more abundant in samples DS and DC than in samples H and V. Both of these observations held true with all four of the cultures selected for this work, and as the four were chosen at random from a long list of cultures in this group, it was

thought that they were fairly representative of the coccus-forming rods.

In just 2 of the 20 tubes was there microscopic evidence of contamination. As a further check on the purity of the other cultures, minute particles of soil from eight of the tubes were transferred to the surface of agar slants and incubated for two days. At the end of the 1st and 2nd days microscopic preparations were made from these subcultures and stained with gentian violet. These preparations verified the evidences of contamination in the two suspected cultures and showed, also, that the cultures appearing pure under the microscope contained no contamination capable of showing upon agar slant cultures from the soil. The subcultures, except from the contaminated tubes, showed the typical morphology of the coccus-forming rods.

The results furnish further evidence that the small coccoid bodies developing in samples DS and DC in Experiment IV (which looked identically like those showing in the present pure culture experiment) may well have been organisms of this type, in which case the interesting inference might be drawn that they took part in the decomposition of the soybean meal.

EXPERIMENT VII

The observation that the pure cultures used in Experiment VI thrived only in the two alkaline or neutral soils suggested that samples H and V might be too acid to support the growth of these organisms. The experiment was repeated, therefore, with two of the pure cultures, but this time an unmeasured quantity of $CaCO_3$ was added to samples H and V before tubing. Microscopic study showed the presence of the organisms in their coccoid form in this experiment and that they were more numerous in samples H and V than in samples DS and DC.

It was concluded, therefore, that these organisms are not well adapted to growth in acid soils. This may explain the reason that similar coccoid forms did not develop in samples H and V in Experiment IV, altho it does not correlate with the observations made in Experiment V in which all four soils were limed without the development of any great numbers of the small coccoid forms.

CONCLUSIONS

This work may be regarded from two angles, *viz.*, it may be considered as a comparison of the four soils studied, or it may be regarded as a study of a bacteriological method for comparing soils.

Viewed as a comparison between the soils, its conclusions are not very striking. A difference was observed, to be sure, between the bacteria developing in the two acid soils and those appearing in the other two soils. As this difference did not ordinarily show so distinctly when $CaCO_3$ was added to the soil, the inference was that it was not due primarily to any distinction between the kinds of bacteria in the soils but was rather the direct result in most instances of the difference in reaction. There are other simpler methods of determining soil reaction, so in this respect it can hardly be said that the investigation has yielded results of any practical value.

It is interesting to notice, nevertheless, that in one instance (with peptone) the difference between these two groups of soils was evident even in the presence of $CaCO_3$. This suggests that there may be some actual distinction between the flora of these soils which is not entirely overcome by liming. The dissimilarity does not wholly correlate with productivity and its significance is not yet known, but it is a matter of interest.

Significance is thus given to the present work if it be regarded primarily as a study of methods, rather than as a comparison of the four soils investigated. A possible means seems to have been pointed out whereby comparisons may be made between the bacteriological properties of different soils which may well lead to more valuable information in the future. The adaptation of Winogradsky's method used in this laboratory is presented, therefore, in the hope that others may make use of it and learn whether it has practical value.

At the same time it must be emphasized that the writers have no prejudice against the plate method of studying soil bacteria. These soils were all plated and the colonies studied before beginning the work described in this bulletin. From this preliminary study much interesting information was obtained. It is felt that the microscopic method should supplement, not replace, the plate method.